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Transformation génétique de l'hévéa

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Studies of the genetic transformation in rubber tree in order to develop a genetic engineering approach for studying latex metabolism

Genetic engineering in rubber tree
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RESUME

La mise au point de la transformation génétique des cals friables embryogènes d'hévéa par *Agrobacterium tumefaciens* s'est heurtée à des problèmes de faible niveau de transformation et de réactions importantes du matériel végétal au stress subit lors de la transformation. Une augmentation significative de l'activité β–glucuronidase (GUS), protéine codée par le gène rapporteur *gusA*, et donc du niveau de transformation a été obtenue à la suite d'une pré-culture des cals friables et des agrobactéries dans un milieu de prolifération des cals sans calcium. L'apport de régulateurs de croissance dans ce même milieu a stimulé l'activité cellulaire des tissus pré-cultivés et de plus forts niveaux d'activité GUS ont été enregistrés.

Les travaux présentés dans ce rapport ont montré que le stress survenant lors de la transformation, même réduit au minimum, engendrait systématiquement le brunissement des tissus et la croissance des agrobactéries. Toutefois, la stimulation de la reprise de croissance des tissus transformés a pu être obtenue par 2 moyens. Le premier a consisté à augmenter l'apport de régulateurs de croissance dans le milieu de décontamination. Cela a permis la prolifération des tissus sans brunissement ni contamination dû à la présence des agrobactéries dans les cals. Cependant, l'activité GUS chutait lorsque l'apport exogène en régulateurs de croissance augmentait. Le second a nécessité le repiquage des cals transformés sous forme de petits agrégats cellulaires. Dans ce cas, une très forte reprise de croissance des tissus a été notée ce qui a permis de supprimer la phase d'élimination des agrobactéries par lavage.

L'activité GUS a été observée dans des petits amas cellulaires après 6 mois de culture. Toutefois, le taux de prolifération de ces amas reste faible et limite la phase d'établissement des lignées cellulaires transgéniques qui seront utilisées pour la régénération de plantes. Nous allons donc focaliser nos études sur les facteurs limitant le développement des cellules transgéniques.

MOTS CLES

Genetic transformation, *Hevea brasiliensis*, *Agrobacterium tumefaciens*

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1. COMPTE RENDU SYNTHETIQUE DES ACTIVITES 1999-2000

Le programme de recherche sur la transformation génétique de l'hévéa mené au Cirad est réalisé en Thaïlande au Kapi (Kasetsart Agricultural and Agro-Industrial Product Improvement Institute) dans le cadre d'une collaboration avec l'université de Kasetsart et le *Rubber Research Institute of Thailand.* L'objectif de ce programme vise à développer une technique de production de plantes transgéniques afin d'étudier le moyen de contrôler l'expression d'un transgène et de caractériser des gènes d'intérêt agronomique.

Le procédé de transformation génétique initié s'appuie sur la méthode de régénération par embryogenèse somatique développée au Cirad à partir de cals friables entretenus à long terme et le processus de transfert de gène par *Agrobacterium tumefaciens* (Schéma 1). Deux facteurs limitant le développement de ce procédé de transformation ont été mis en évidence : la faible efficacité de transformation du système *Agrobacterium*, et la dégénérescence des tissus que ce dernier induit.

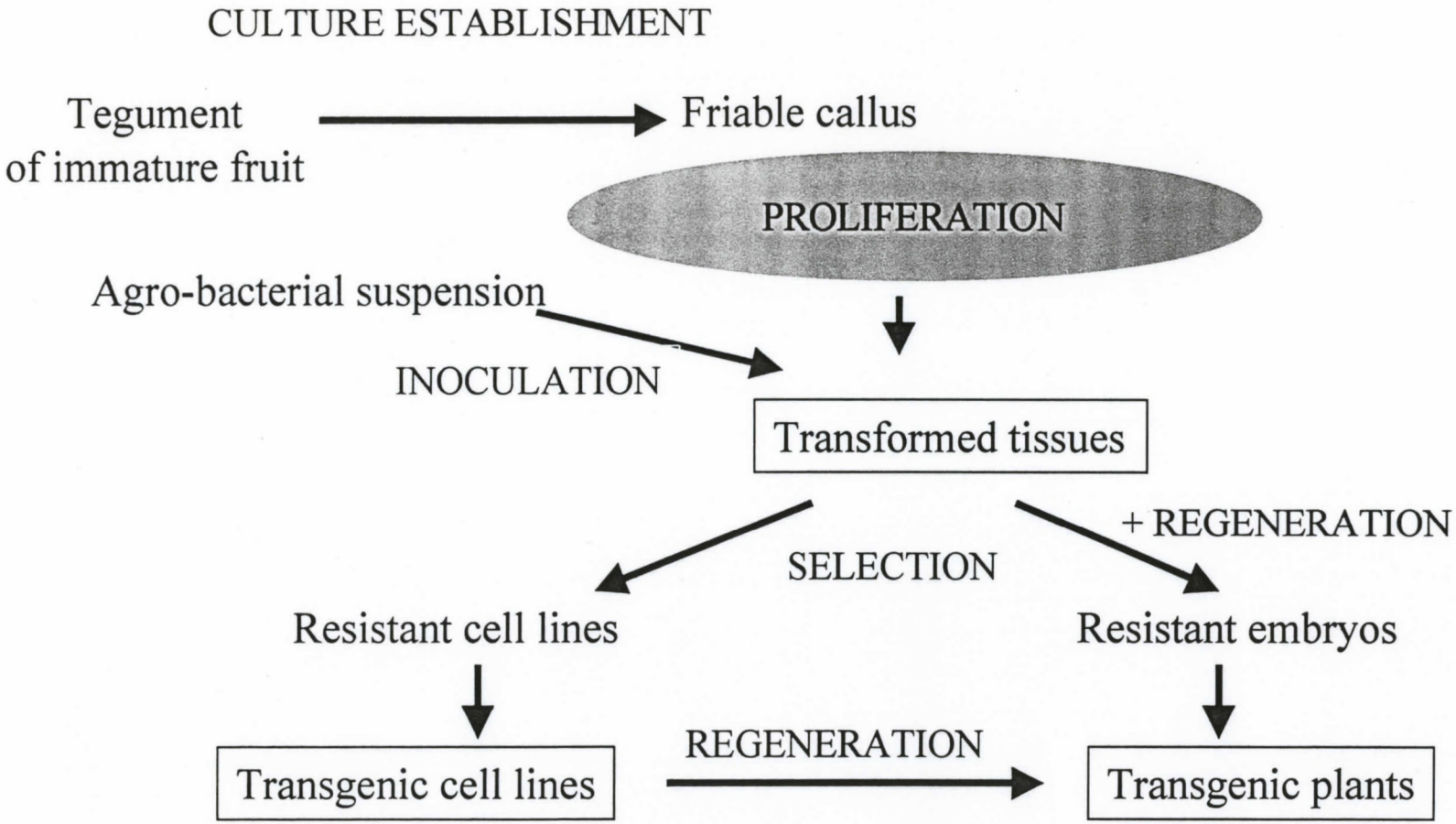
La pré-culture du matériel végétal et des agrobactéries dans un milieu sans calcium s'est avéré être un conditionnement déterminant pour augmenter l'activité transitoire du gène rapporteur *gusA* dans les tissus transformés (cf. rapport annuel 1998-99) . Dans ces conditions de pré-culture, l'augmentation de la concentration en régulateurs de croissance stimule aussi l'activité GUS dans les tissus transformés laquelle est liée à une forte activité cellulaire observée dans les tissus en fin de pré-culture.

Les efforts de recherche ont porté cette année sur les problèmes de dégénérescence rapide des tissus après transformation et les contaminations par *Agrobacterium* qui s'ensuivent. Nous avons émis l'hypothèse qu'un tissu transformé actif avait plus de chance de se défendre contre l'agression bactérienne et les autres stress liés à la transformation. De ce fait, nous avons étudié les moyens d'une part de favoriser la croissance des tissus, et d'autre part, de réduire les stress survenant lors de la transformation.

1- Les expériences visant à diminuer la pression bactérienne n'ont pas conduit à une amélioration de la croissance des tissus transformés. En effet, dans des conditions douces de transformation, il y a perte de l'activité GUS et le brunissement des cals survient malgré tout rapidement.

   - La souche d'*A. tumefaciens* EHA105 utilisée est considérée comme très virulente et donc agressive contre les tissus végétaux. Cette souche est toutefois la seule pouvant transformer efficacement les tissus d'hévéa. Même dans les conditions optimales de pré-culture, aucune autre souche testée n'a donné des niveaux d'activité transitoire GUS suffisants.
   - Les réductions du temps d'inoculation ou de co-culture, ainsi que de la concentration de l'inoculum affectent sévèrement l'efficacité de transformation et n'ont pas d'effet majeur sur la survie du matériel transformé.

Diagram 1:Strategies for *Agrobacterium tumefaciens*-mediated genetic transformation of friable calli from rubber tree



2- Les facteurs jouant sur la stimulation de la croissance des cals se sont avérés plus efficaces dans la lutte contre le brunissement et les contaminations survenant dans les cultures. La reprise de croissance des tissus transformés est donc un paramètre important à contrôler.

- La croissance des tissus transformés augmente avec la concentration en régulateurs de croissance dans le milieu de décontamination (milieu de prolifération des cals additionné d'un agent bactériostatique, le Ticar^{TM}). Malheureusement, l'activité GUS diminue plus fortement dans les tissus transformés cultivés sur une forte teneur en régulateurs de croissance. Des analyses cytologiques sont en cours afin de déterminer les raisons de cette chute d'activité.
- La culture de petits agrégats d'environ 1 mm de diamètre permet d'obtenir un très fort taux de croissance relative par rapport aux tissus généralement repiqués en amas tissulaire de 5-6 mm. Cette croissance est maintenue lorsque les cals transformés sont repiqués sous cette forme de petits agrégats sans avoir recours à de forte concentration en régulateurs de croissance exogène. Nous pensons que le meilleur contact des tissus avec le milieu de décontamination facilite l'action de l'agent bactériostatique et une meilleure nutrition du cal. Dans ce cas, nous avons pu observer le maintien pendant plusieurs mois de l'activité GUS dans les tissus transformés.

Enfin, des expériences préliminaires ont permis de mettre en évidence que la kanamycine, l'agent de sélection des cellules transgéniques généralement utilisé chez d'autres espèces, ne limitait pas le développement des tissus non transgéniques chez l'hévéa. Par contre, nous avons montré qu'une autre néomycine, la paromomycine, pouvait être utilisée pour la phase de sélection. Des travaux sont en cours pour la définition d'une séquence de milieux permettant la sélection et l'établissement de lignées cellulaires transgéniques.

Bien que l'activité GUS ait été observée pendant plus de 6 mois dans les tissus transformés, cette activité est réduite à de petits amas cellulaires dont les capacités de prolifération semblent réduites. Nos objectifs à court terme sont donc de définir les conditions favorables au développement des cellules afin d'établir des lignées cellulaires qui serviront à la régénération clonale de plantes transgéniques. Pour cela, une étude cytologique approfondie de l'influence des facteurs hormonaux entre autres et la définition de séquences de sélection adaptées aux tissus d'hévéa va être entreprise.

2. PERSPECTIVES DU PROGRAMME DE GENIE GENETIQUE

2.1 Présentation du dispositif actuel

Le programme de génie génétique chez l'hévéa développé au Cirad s'appuie sur la recherche de gènes d'intérêts à partir des études en agro-physiologie moléculaire et le développement d'une méthode de transformation génétique. Ces opérations de recherche sont menées conjointement au CIRAD-Montpellier (Ecotrop et Biotrop) et en Thaïlande (Kasetsart-RRIT-Cirad). La composante transformation génétique a été initiée au Kapi à l'université de Kasetsart où nous pensions amplifier notre action dans

le futur. Etant donné le désengagement du Kapi (cf. Note du 12 avril 2000), un retour au Cirad-Montpellier de ce programme a été planifié pour juillet 2001.

Le programme de transgénèse a été initié dans le but d'utiliser ce nouvel outil d'analyse pour décortiquer les mécanismes moléculaires liés à la productivité chez l'hévéa et d'offrir des possibilités d'application par la modification génétique spécifique de clones améliorés par voie de sélection classique. Cela passera donc par une bonne connaissance du fonctionnement physiologique et de la régulation des gènes impliqués dans la production de caoutchouc, le clonage des gènes à modifier ou bien l'utilisation de gènes hétérologues, et la transformation génétique du clone étudié.

2.2 Les différentes étapes du procédé de transformation en cours de développement

Les différentes étapes du procédé de transformation en cours de développement chez le clone PB 260 sont décrites dans le schéma 2. Le temps nécessaire pour la production de plantes transgéniques est estimé à environ 28 mois à partir d'une lignée cellulaire embryogène établie. Ce délai implique donc une certaine lourdeur de l'approche transformation et justifie donc toute optimisation du procédé qui pourra raccourcir les différentes étapes et diminuer les effectifs à analyser.

D'ores et déjà, nous pouvons penser que l'obtention de lignées transgéniques représente un atout considérable quant à la possibilité de les cribler, avant de les transférer sur les milieux de régénération, pour leur nombre de copies de transgène intégré, et de caractériser de façon précoce les niveaux et l'impact de l'expression du transgène sur la fonction étudiée. Etant donné le délai de régénération de plants transgéniques, le choix de lignées n'ayant qu'une copie du transgène et l'exprimant correctement permettra de réduire les effectifs et de faciliter les analyses au niveau de la plante entière. En outre, la lignée transgénique pourra représenter un outil pour des études avancées en laboratoire.

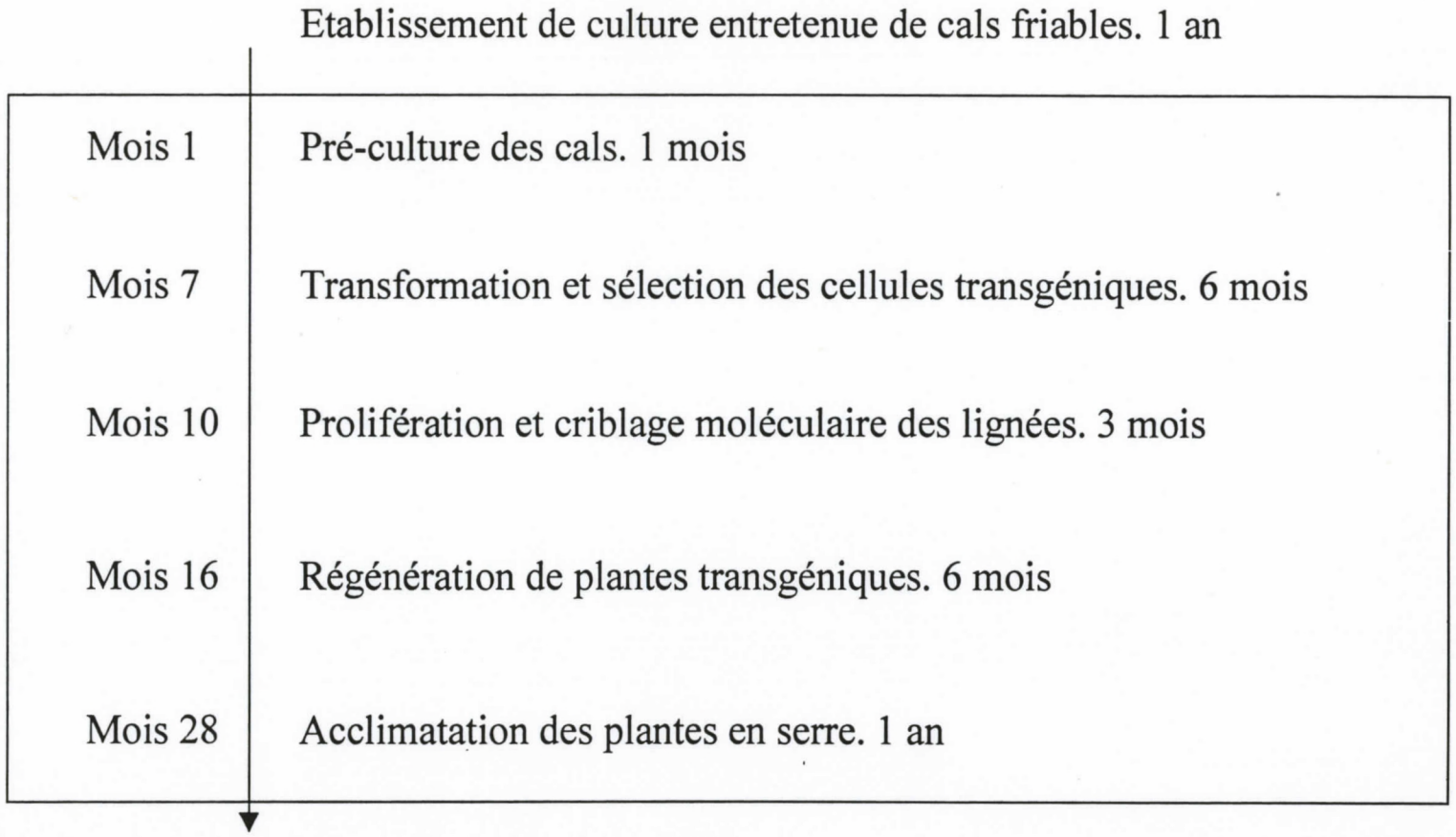
2.3 La validation et l'optimisation du procédé de transformation génétique

Le procédé de base de transformation génétique devra être valider par la production et la caractérisation moléculaire de plantes transgéniques chez le clone PB 260. Des travaux d'optimisation du procédé devront être maintenus avec un axe d'amélioration des différentes étapes et un axe d'utilisation de nouveaux vecteurs de transformation. Cette dernière voie touche principalement l'amélioration des phases de sélection et d'expression des transgènes.

Utilisation d'un gène rapporteur codant pour une protéine fluorescente
Le remplacement de *gusA* par *gfp* (gene encoding green fluorescent protein) devrait permettre un criblage des cellules transgéniques sur du matériel vivant exprimant des protéines fluorescentes. Cette détection facilitera d'une part l'observation de l'expression des transgènes au cours du processus, et d'autre part la sélection des cellules végétales pour la création de lignées transgéniques.

Utilisation de gènes de sélection comportant un intron
La présence d'un intron dans la séquence du gène de sélection permettra d'éliminer plus efficacement les agrobactéries qui n'exprimeront plus le gène de résistance en question.

Schéma 2. Estimation de la durée des différentes étapes du processus de transformation génétique chez l'hévéa.



De ce fait, nous réduirons le stress biotique permanent exercé sur les cellules végétales qui peut induire une différentiation cellulaire c'est à dire une perte d'activité des cellules transformées et surtout une perte de leur capacité embryogène.

Utilisation des séquences MARs pour améliorer la stabilité de l'expression des transgènes
L'introduction de séquences associées à la matrice nucléaire (MARs) de part et d'autre des transgènes permet en général de réduire les écarts d'expression des transgènes entre des plantes issues de différents événements de transformation. Ces variations sont liées au site d'insertion dans le génome. Par conséquence, cela devrait permettre de réduire le nombre d'individus à étudier et ainsi alléger fortement le dispositif à mettre en place pour la caractérisation d'une fonction.

2.4 Les applications possibles avec des gènes d'intérêt agronomique

Les modifications génétiques en vue d'une amélioration de la productivité des hévéas devront être appropriées à chaque clone, cela en relation avec ses propres caractéristiques et limites de production. Les recherches qui ont conduit à la typologie clonale et les données des études de physiologie-moléculaire doivent permettre dès aujourd'hui de définir les limites de certains clones par exemple ayant une faible tolérance au stress impliquant une sensibilité à l'encoche sèche ou un faible potentiel de production lié à une faible teneur en saccharose intra-laticifère.

La modification des teneurs en protéines jouant un rôle dans les mécanismes de résistance au stress oxydatif, la coagulation des particules de caoutchouc, etc... doit de plus être réalisée avec prudence sans perturber le bon développement de la plante tout entière. Pour cela, il a été développé une stratégie visant à restreindre l'expression des transgènes en utilisant des séquences de régulation de la transcription ou promoteurs.

Ainsi, les premières applications de la transformation génétique de l'hévéa pourront porter sur :

Le contrôle de l'expression du transgène dans les tissus laticifères
Utilisation du promoteur du gène codant pour l'hévéine. La présence spécifique de cette protéine a été démontré par immuno-localisation. L'expression du gène correspondant est donc supposée se faire spécifiquement dans les cellules laticifères.

Le contrôle de l'expression du transgène par inductibilité
Utilisation du promoteur du gène codant pour une des enzymes à activité glutamine synthétase. L'expression des gènes *gs* et l'activité de cette enzyme sont augmentées dans le latex d'arbre stimulé par l'éthrel.

- La diminution des problèmes d'écoulement
Résistance au stress par sur-expression des facteurs de détoxication, et action sur le mécanisme de la coagulation.
L'extinction de l'expression des gènes codant pour des protéines impliquées dans le processus de coagulation, liaison des particules de caoutchouc par l'hévéine et son récepteur IW3 situé à l'extérieur de la membrane des particules, et bien d'autres facteurs de neutralisation des charges telle que les glucanases pourrait être obtenue. Une

attention particulière devra être prise sur cette dernière option car ce sont aussi des facteurs de résistances aux pathogènes.

La stimulation du chargement en saccharose dans les tissus laticifères
Pour certains clones, les faibles teneurs en saccharose intra-laticifère pourraient être expliquées lorsque le métabolisme ne l'utilise pas, par un blocage à différents niveaux ou des déviations des voies de l'alimentation en carbohydrate. Bien que seules les études en éco-physiologie et électrophysiologie de l'hévéa pourront élucider ces points de blocages, quelques hypothèses pourraient être émises quant à la modification spécifique de certaines enzymes connus pour être responsable :

- de la répartition des carbohydrates au sein des tissus puits pour la croissance, le stockage, la production de fruits ou de caoutchouc,
- du transport du saccharose,
- ou au niveau du chargement ou de la transformation du saccharose dans le système laticifère lui-même.

Enfin, il doit être souligné la nécessité d'une interaction de programmation entre les différentes opérations embryogenèse somatique, transformation génétique et physiologie-moléculaire. En effet, la typologie clonale de fonctionnement métabolique du système laticifère en liaison avec les connaissances sur le plan physiologique et d'expression génique orienteront le choix des clones et des gènes candidats pour une transformation génétique. Cette demande spécifique pour la modification génétique d'un clone impliquera des travaux d'adaptation de notre maîtrise des procédés en matière de culture *in vitro* et de transfert de gène.

2.5 Le partenariat en Asie

Le développement de notre partenariat en Asie dans le domaine des biotechnologies revêt plusieurs intérêts :

- Mettre en place une collaboration avec des organisations des pays producteurs de caoutchouc telle que le RRIT en Thaïlande pour favoriser notre connaissance en aval ;
- Mettre en place des collaborations scientifiques pour augmenter notre potentiel de recherche et pour faciliter la validation des outils développés et leurs applications sur place ;
- Obtenir une ouverture sur un projet régional afin d'acquérir des financements auprès de l'UE. Dans ce cas, la recherche d'un deuxième partenaire asiatique est nécessaire. Les équipes du KUMHO en Corée, du RRIM en Malaysie ou de certains laboratoires en Chine (cf. Note Mynard, août 1999) représentent autant de perspectives de collaboration.

En outre, la mise en place d'un réseau régional ou international dans le domaine des biotechnologies chez l'hévéa aurait pour avantages de mieux identifier des partenaires potentiels pour le montage de projets de recherche, de faire ressortir les priorités en matière de recherche, et d'évaluer l'impact de leurs applications.

GENETIC ENGINEERING IN RUBBER TREE

1. GENERAL INTRODUCTION

The research programme scheduled for the period of July1998 to June2001 in genetic engineering in rubber tree deals with the setting up of the *Agrobacterium tumefaciens*-mediated genetic transformation procedure. This biotechnology will represent a powerful tool to study the regulation of the expression of genes involved in the latex production and consequently to define candidate genes for genetic modification of specific clones. The present challenge consists in setting up the genetic transformation procedure in rubber tree, a perennial crop which was long considered as recalcitrant to vitro-culture.

2. PRESENTATION OF THE GENETIC TRANSFORMATION APPROACH

The genetic transformation procedure developed on rubber tree in this project uses *Agrobacterium tumefaciens*-mediated gene transfer system and plant regeneration by somatic embryogenesis from friable calli.

This process consists of several steps:
- Pre-cultivation of the plant material and *Agrobacterium tumefaciens* strain;
- Inoculation of friable calli with *Agrobacterium* suspension;
- Co-cultivation;
- Decontamination of the transformed tissues;
- Selection of transgenic cells and establishment of transgenic cell lines;
- Regeneration of transgenic plants;
- Analysis of transgenic plants.

In this process, 2 determining factors had impeded to bring to a successful conclusions: the low transformation efficiency and the concomitant necrosis and contamination of tissues after transformation. For that, we collected data as morphological parameters of tissue (growth rate, tissue browning, percentage of contamination) and GUS activity by histochemical assay (the efficiency of the gene transfer process has been assessed by recording the number of cell revealing a transient GUS (β-glucuronidase) activity encoded by the *gusA* reporter gene). Some cytological analyses have also been performed in some tissue, on the one hand to describe cell characteristics which were related to the transformation competency, and on the other hand to study the effect of the modification of both medium and environmental factors. These studies were carried out at Cirad-Montpellier in the framework of training supported by the French embassy in Bangkok.
Thus, our experiments have been conducted according to the strategies developed here:

1- Study of factors allowing high transformation efficiency without taking into account any effect on the tissue. Indeed, drastic transformation conditions have been used to obtain significant increase in transient GUS activity and thereby determine or underline the important factors for transformation.

2- Study of factors stimulating the growth recovery of transformed tissues. Agrobacterium cells stress plant cells and induce their degeneration. In order to insure that basic transformation conditions allow the survival and the growth of a large number of transgenic cells, we have to improve the proliferation potential of these tissues. This potential is essential for developing transgenic cell lines by applying selection.

3. IMPROVEMENT OF THE TRANSFORMATION EFFICIENCY

The development of the genetic transformation procedure had been impeded by a low number of transformation events per experiment. Calcium supply was determined as the main limiting factor of the gene transfer process. A decrease in calcium in the culture medium allowed a dramatic increase in the transformation efficiency [3, 4].

The researches conducted over the first year of the present project led us to characterise the effect of $CaCl_2$ in the plant and bacterium culture media [5]. Depletion of $CaCl_2$ in the plant culture medium before transformation increased both transformation efficiency, recorded as transient GUS activity, and cell activity related to a important presence of undifferentiated and embryonic cells in the tissue [6]. A decrease in the calcium content was also noted in these friable tissues. A phase of pre-cultivation of friable calli on maintenance medium without $CaCl_2$ before transformation is essential to obtain high transformation efficiency. Moreover, the presence of $CaCl_2$ in the inoculation medium (*Agrobacterium* suspension in plant culture medium) reduced also the GUS activity that might be related to a direct inhibition of the gene transfer process occurring in the *Agrobacterium* cell.

An increase of the concentration in growth regulators (GR) has also enhanced transformation efficiency. We assumed that plant growth regulators might play a role by increasing plant cell competency for transformation. Unfortunately, cytological studies did not provide us such a correlation between cell characteristics and GUS activity (Rattana and Teinseree, 1999 cited in [5]). Besides, 1 mg/l GR in the pre-cultivation medium reduced tissue browning which could be related to an improvement of the tissue stress tolerance.

4. PROLIFERATION OF TRANSFORMED TISSUES

We have noticed that no contamination occurred when tissues had a good growth. Endogenous plant cell defence could fight *Agrobacterium* overgrowth. Moreover, we assumed that if all transgenic cells can proliferate, we increase the chance to get stable transformation and that will enable the development of transgenic cells. For that, a decrease in *Agrobacterium* pressure (transformation conditions) has been performed while maintaining sufficient transformation efficiency. Besides, conditions of *Agrobacterium* elimination have been improved. Finally, the effect of the selection step on transgenic cell development has been studied, in particular because the proliferation of non-transgenic cells can compete with the transgenic ones.

The removal of limiting factors for the *Agrobacterium tumefaciens*-mediated gene transfer process allowed us to favour the development of transgenic cells for further

selection and creation of transgenic cell lines able to regenerate plantlets. In this aim, we studied the influence of the main transformation factors on the growth rate of transformed tissues in order to reduce non-necessary stresses upon transformation, which could induce tissue browning.

The below research topics have been implemented for the following purposes:

* Decrease the *Agrobacterium* pressure while maintaining sufficient transformation efficiency:
  - use of less virulent Agrobacterium strain;
  - optimise the duration of inoculation;
  - studying secondary factors such as acetosyringone supply (virulence gene inducer).
* Stimulate plant cell growth
  - quality of plant cell line;
  - growth regulators (GR).
* Development of an adapted tissue culture system for selection of the transgenic cells thanks to a better contact with the medium containing the selective agent (antibiotics).
* Determination of culture conditions for selection (preliminary results):
  - source of antibiotics, concentration and timing of application.

4.1 Decrease the *Agrobacterium* pressure while maintaining sufficient transformation efficiency

4.1.1 Use of a less virulent *Agrobacterium* strain

Effect of Agrobacterium strains on the transformation efficiency and the growth recovery of inoculated friable calli (P1)

Gene transfer efficiency has been improved by the use of a pre-cultivation step of friable calli on a $CaCl_2$ –less medium and the use of a bacterial re-suspension $CaCl_2$ – less medium as well. This procedure has been set up on the virulent EHA105 strain containing binary plasmid pC2301. The aim of this experiment is to check on the one hand if this new procedure increase the transformation efficiency in several *Agrobacterium* strains and on the other hand if the negative effect of the virulent EHA105 strain (difficulties to eliminate bacteria, tissue browning) can be decreased by the use of less virulent *Agrobacterium* strains.

Table 1. Gus activity of infected tissue recorded 6 days after co-cultivation

Treatment	No. Of Calli	FM (mg/aggr.)	GUS activity	
			No spots/aggreg.	No spots/gFM
C58pMP90pC2301	30	46±2	0[a]	0[a]
AGL1pC2301	30	-	0[a]	0[a]
LBA4404pC2301	30	-	0[a]	0[a]
GV2260pC2301	30	52±0	0.1±0.0[a]	1.1±0.3[a]
EHA105pC2301	30	49±7	5.9±1.9[b]	120±35.9[b]

[1] Fisher test (LSD). All values with the same letter are not significantly different (P <0.01).
[2] Each value is the mean of 5 repetitions.
nd: not determined.

High level of GUS activity was recorded only in tissue inoculated with EHA105pC2301 (Table 1), however the 120 blue spots/gFW represents a lower efficiency than those commonly observed (about 200 to 300). A low calli competence is assumed to be responsible for it. In the case of transformation with EHA105, an average of 6 blue spots per cell aggregate has been observed.
Two blue spots have been observed with treatment GV2260, and 5 calli out of 103 have been contaminated 6 days after co-cultivation with this *Agrobacterium* strains. There is no direct relationship between a high transformation efficiency and presence of bacteria in the calli.
With regards to tissue browning, tissues inoculated with LBA4404 were less brown than other with other strains, but unfortunately no blue spots have been observed here.

Table 2. Morphological parameters recorded 18 days after co-cultivation on infected cell aggregates cultivated on a range of 3-4,D and BAP concentrations (growth : 2+ = low, 3+ = common, 4+ = good ; browning 1+ = light-yellow and no blown, 2+ = more yellow and include 2-3 brown spots in calli) before subculture to MD3.

Treatment	No	Growth (%)				Browning (%)				Contamination
	Aggregates	1	2	3	4	1	2	3	4	No
C58pMP90pC2301	94	6	56	48	0	96	51	39	0	0
AGL1pC2301	95	63	34	3	0	0	13	83	4	2
LBA4404pC2301	82	11	67	21	1	18	33	49	0	0
GV2260pC2301	91	36	57	7	0	8	22	70	0	0
EHA105pC2301	102	5	75	20	0	9	20	71	0	0

Agrobacterium strains affected differently tissue growth rate and browning (Table 2). Tissues infected with GV2260 and LBA4404 have got the lowest growth rate and the highest browning level with EHA105.
The transformation efficiency of different bacterial strains was assessed on friable calli by histochemical analysis. Two out of five strains tested showed GUS activity 6 days after co-cultivation. However, GUS activity recorded with strain C58pGV2260 was very low and only EHA105 showed up a sufficient transformation efficiency to consider following evolution of transgenic cells. In addition we confirmed the importance of calcium-less media both for bacterium and plant cell cultures to get high transformation efficiency with this strain.

4.1.2 Effect of the inoculation

Effect of the duration of immersion of friable calli with Agrobacterium suspension on the transient GUS activity and the tissue growth recovery (R4)

Subculture of small aggregates after co-cultivation has been shown very efficient in terms of growth recovery, and fighting browning and contamination (see § below). In addition, this size of aggregates does not require a washing step to eliminate *Agrobacterium* cells. The better contact of aggregates with the medium containing antibiotics stopped drastically bacterial overgrowth. Besides, we have shown in the preliminary experiment N2 that transient GUS activity of cell lines 95PB and 98PB increased respectively from 178±126 to 288±205 spots/gFM and from 57±45 to 88±30 spots/gFM when calli are inoculated for 30 seconds instead of only one. However, no difference has been noticed at statistical level (LSD at 95%).
This experiment aimed at increasing GUS activity by extending the time of contact of Agrobacterium cell suspension with friable calli (inoculation) without inducing any water stress due to plant tissue immersion. In addition, cefotaxime has been removed in order to reduce its well-known negative effect on plant tissue. For that reason, infected cell aggregates have been observed along the culture and no selection has been applied

for 2 months. At this time, GUS activity has been performed and relative growth rate recorded in order to assess the long-term effect of each treatment.

Table 3. Gus activity recorded 6 days after co-cultivation on infected tissue subcultured as small size aggregates (1mm).

Inoculation	Aggregates	FM[1,2]	GUS activity		Browning	Contamination
(sec)	No	.mg/agg	Spots/agg[1,2]	Spots/gFM[1,2]		(%)
0	20	6.23	0	0	+	0
1	100	4.1±0.9[a]	0.3±0.4[ab]	73±85[ab]	++	0
10	100	3.9±0.7[a]	0.2±0.3[ab]	49±62[ab]	+++	0
30	100	4.4±0.4[a]	0.3±0.5[ab]	79±124[ab]	+++	3
60	100	4.2±0.4[a]	0.7±0.6[a]	164±121[a]	+++	1
180	100	6.4±0.9[a]	0.2±0.2[ab]	33±33[b]	++++	4

[1]Fisher test (LSD). All values with the same letter are not significantly different (P<0.01).
[2]Each value is the mean of 5 repetitions.
nd: not determined.

Infected tissues were transferred as small aggregates onto MD1. Aggregates from all treatments had a similar weight (Table 3). GUS activity increased with the time of inoculation to reach 164 blue spots/gFM for1 minute of inoculation and then decreased for longer time. Interestingly, tissue browning increased also with the time of inoculation, and thereby might explain the drop of GUS activity for 3 minutes of inoculation for which tissue were necrotic.

Despite an increase of the transient GUS activity by applying longer time of inoculation, tissue browning occurring upon transformation did not allow to detect any activity after one month on decontamination medium. This problem must be taken into consideration whether we want to consider development of stable transformation. Besides, this experiment should be repeated in order to confirm effects both of non-washing when small aggregates are transferred onto MD1 and of removal of cefotaxime into MD1 which could play a role to reduce tissue browning.

4.1.3 Study of acetosyringone supply (virulence gene inducer)

Effect of the duration of the acetosyringone supply into the bacterial suspension on the transformation efficiency of friable calli (R1)

Induction of *Agrobacterium* suspension with 100 µM acetosyringone has been shown absolutely necessary to keep a high transformation efficiency [5]. A variation of the efficiency between experiments has been noticed and might be related to a variation in the time of AS induction of *Agrobacterium* cells.
This experiment aims at emphasising the real effect of the duration of AS supply.

Table 4. Gus activity recorded on cell aggregates 6 days after co-cultivation

AS induction	Aggregates		GUS activity	
Hours	No	mg/agg	Blue spots/aggr	Spots/gFM
0		44±5[bc]	0.9±0.6[a]	20±13[b]
Resuspension	35	39±6[c]	1.3±0.7[a]	33±19[a]
5	35	55±8[ab]	1.4±1.1[a]	25±21[b]
21	35	65±7[a]	0.4±0.4[a]	7±6[c]
29	35	65±13[a]	0.9±0.3[a]	14±3[b]
45	35	52±11[b]	0.9±0.9[a]	19±22[b]

[1] Fisher test (LSD). All values with the same letter are not significantly different (P<0.05).
[2] Each value is the mean of 5 repetitions.

Addition of AS into the resuspension medium increased significantly the number of blue spots per gFM but conversely a longer application decreased significantly the GUS activity and then increased again. The significance of this discrepancy between the positive and the negative effect of AS induction must be analysed cautiously being given the low efficiency of transformation in this experiment (100-200 spots/gFM) are commonly recorded in other experiment). In fact, we observed in several previous experiment an increment of GUS activity after 21 hours of AS and here a decrease has been recorded. A low ability of friable calli to be transformed might be responsible of this discrepancy.

EHA105pC2301 cells have been induced by 100 µM acetosyringone for 0 to 45 hours in order to optimise virulence induction for rubber tree friable calli transformation.
Transformation efficiency of this experiment was lower than usual (with a maximum of 33 blue spots/gFM instead of 100-300). Despite this, it has been shown that a short induction of *Agrobacterium* cell has given the highest GUS activity.
The use of brief acetosyringone induction seems to be an optimum for the transformation rubber tree friable calli with EHA105pC2301.

4.2 Stimulation of the plant cell growth

4.2.1 Dependence on the plant cell line characteristics

Effect of the age of the rubber tree cell lines on the transformation efficiency and the growth recovery of transformed tissue (R2 and R8)

Experiment1:
Transformation efficiency recorded on rubber tree cell line 95-PB260 is not consistent along the year. Recent data showed up that this cell line has to be stimulated by 1 mg/l growth regulators to maintain high transformation efficiency. A loss of cell activity of this old cell line is supposed to be the main reason of the decrease of the efficiency.
For that reason, we compared the transformation competency of 2 and 5 year-old cell lines respectively established in 1998 and 1995 that are named 98PB and 95PB.

Table 5. Gus activity of aggregates 6 days after co-cultivation.

Cell line age	Aggregates		GUS activity	
Years	No	FM (mg/aggr)[1,2]	Blue spots/aggregate	Blue spots/gFM
5	30	38.6±12.6[ab]	5.7±1.7[ab]	178±126[ab]
2	30	49.3±11.0[a]	2.6±2.1[b]	57±45[b]

[1] Fisher test (LSD). All values with the same letter are not significantly different (P <0.05).
[2] Each value is the mean of 5 repetitions.

Table 6. Morphological aspects of transformed cell aggregates 12 days after co-cultivation

Cell line age	Inoculation	No	Growth (%)				Browning
(years)	(sec)		1	2	3	4	(%)
5	0	4	0	0	25	75	0
5	1	10	50	30	20	0	0
2	1	10	50	50	0	0	100

A higher GUS activity has been obtained with the cell line established in 1995 than 1998 (Table 5).
Two after co-cultivation, we can see that inoculation affected the growth rate for the cell line 95 but did not induce any browning on the transformed tissues (Table 6).

Conversely, both growth rates of tissues from cell line 98 were dramatically reduced and consequently tissue browning occurred on 100% of cell aggregates in culture.

Cell characteristics of the cell lines are supposed to be the determining factor for transformation competency and subsequent growth recovery of transformed tissue. Tissue growth is also essential to fight the browning that occurred in non-active tissue.

Experiment 2:

A drop of GUS activity has been recorded since small aggregates are commonly transferred onto medium of selection MD1. This decrease might be explained for one thing by a loss of plant cell competency. As matter of fact, transformation efficiency of old cell line 95PB is variable from one experiment to another and even tends to decrease despite the improvement of friable calli pre-cultivation conditions. Thus, 5 and 2-year-old cell lines established respectively in 1995 and 1998 have been compared.

Table 7. GUS activity recorded on small aggregates 6 days after co-cultivation of friable calli (95PB and 98PB) with EHA105pC2301.

Aggregates				GUS activity[1,2]		Browning	
Age (years)	Size (mm)	No	FM[1,2] (mg/agg)	No blue spots/agg	No spots/gFM	Before GUS	After GUS
5	1	30	Nd	0[a]	0[a]	+	+
5	1	30	1.8±0.2[a]	0.90±0.97[a]	512±555[a]	++	++/+++
2	1	30	1.5±0.1[a]	0.09±0.10[a]	60±70[a]	++	++++

[1]Fisher test (LSD). All values with the same letter are not significantly different (P<0.01).
[2]Each value is the mean of 5 repetitions.
nd: not determined.

Two cell lines of friable calli established in 1995 and 1998 have been inoculated with EHA105pC2301. The GUS activity recorded 6 days after co-cultivation on small aggregates from 2 and 5-year-old cultures was respectively for the 2 cell lines 60±70 and 512±555 blue spots/gFM (Table 7). The 2-year-old culture turned very brown after GUS incubation and was contaminated by *Agrobacterium*. On the contrary, the transformed 5-year-old culture could stand incubation and no contamination has been observed. This previous observation was confirmed by morphological observation made at day14 where transformed 2-year-old tissues were totally brown in culture (Table 7).

Table 8. Morphological aspects of small aggregate 14 days after co-cultivation of friable calli with EHA105pC2301. T1 (95PB) ; T3 (98PB).

Cell line age	Aggregates*		Growth	Browning I^{ary}-II^{ary} tissue
(years)	Size (mm)	No		(%)
5	1	25	3 / 4+	68-0
5	1	150	2 /3+	39-0
2	1	150	1/ 2+	100-83

* no contamination observed

Table 9. Morphological aspects of transformed tissues after selection on 50 mg/l paromomycin. Friable calli were inoculated with EHA105pC2301 for 1 second and co-cultivated for 2 days. T1 (95PB) ; T3 (98PB).

Days after co-cultivation	Cell line age	Initial Aggregates		GUS activity on non-resistant aggregates
	(years)	No	No resistant	No blue units
28	5	135	121	Nd
	2	150	31	Nd
49	5	137	83	Nd
	2	24	13	Nd
63	5	66	49	Nd
	2	10	8	Nd
77	5	29	20	Nd
	2	9	13	Nd
103	5	17	22	Nd
	2	9	14	Nd
117	5	17	0	Nd
	2	6	0	Nd
125	5	16	0	2 blue clusters
	2	6	0	2 blue spots+2 clusters

Nd: non determined

Three subcultures with selective agent were necessary to reduce the number of resistant tissues. After 125 days of selection, no resistant has been observed. GUS assay performed on these tissues showed that transgenic units were still alive but not active, the 2 blue spots and 4 small cell clusters revealing a problem of development of these transgenic cells.

The transient GUS activity is likely to be dependent on the plant cell tissue characteristics. In this experiment, transformation efficiency was higher with 5-year-old than 2-year-old culture but other experiments have shown inverse outcome. Thus, we suggest that transformation depends more on cell characteristics that change along the culture for a same cell line. Ability of the cell line to resist to transformation stress is an important factor to have plant tissue growth recovery. Indeed, 2-year-old cell line was particularly prone to browning (browning upon GUS incubation and then along the first subculture). Thereby, this cell line could not stand selection pressure and all tissue turned rapidly necrotic. In contrast, 5-year-old culture had a good growth recovery on the first decontamination medium without selection pressure. Consequently, selection of transgenic cells has been applied in right condition. Unfortunately, no resistant cell line has been established after 100 days of selection. Either the procedure of selection was not adapted to rubber tree tissue or transformation efficiency was not high enough to allow to some transgenic cells to be developed. Although all tissues turned brown 125 after co-cultivation, some GUS activity has been recorded. After more than 3 months of selection, transgenic units were still not developed (2 spots and 4 small cell clusters), which can explain the fact we did not get any resistant tissues. Development of transgenic cell remains now the main problem to be solved to go ahead with genetic transformation procedure.

4.2.2 Effect of the exogenous growth regulators

Effect of growth regulators both concentration and timing in the decontamination medium on growth recovery of inoculated friable calli (W9)

According to the experiment W1 and W3 [5], an increase of 3,4-D and BAP concentrations improves callus growth recovery either in the pre-culture medium at 1

mg/l or in the decontamination medium at 1 or 2 mg/l. The aim of this experiment is to confirm their positive effect and to determine the optimal timing of application of a high GR.

The use of pre-cultivation period of friable calli onto medium containing 1 mg/l GR significantly increased GUS activity. Although growth rate recorded at the end of the pre-cultivation increased with the GR concentration, no real effect of pre-cultivation conditions has been observed on growth and browning after the co-cultivation of plant tissue with *Agrobacterium* cells.

The increase both of concentration and timing of GR in the medium of decontamination (MD) stimulated growth recovery of tissue after transformation and struggled or delayed tissue browning. However, extreme application (3 subcultures with 2 mg/l GR) had an opposite effect.

The GUS activity of infected tissue tended to decrease along the post-cultivation period (observation from day6 to day 60). This drop became more pronounced when concentration and timing of GR application increased. This loss of GUS activity might be related either to a transgene silencing leading to a *gus*A or *npt*II gene extinction or a loss of activity and then death of stressed transgenic cells (day17, 40-95% cell of tissue turned brown). However the *nptII* extinction might not play any role to transgenic cell death because no selection phase was applied until day34. Another hypothesis might be a competition between transgenic and non-transgenic cell development. Indeed, application of high GR concentration boosted cell division and non-transgenic cells might be favoured by this treatment.

Nevertheless, observation of the development of blue cell clusters and cell masses (day34) from blue spots (day6) gave evidence that transgenic cells have been able to divide. Transgenic cell division means that these cells are alive and this reinforce the hypothesis of transgene silencing occurring when high GR concentration are used. Transgene silencing was shown to be mediated by T-DNA methylation. The transgene silencing hypothesis will be verified by application of an anti-methylation agent (5-Azacytidine) in next experiment.

Table 10. Effect of the application of growth regulators on tissue browning, their relative growth rates (RGR) and their GUS activity 17, 34 and 60 days after co-cultivation. Inoculation of friable calli and transfer of 3 mm aggregates. Transient GUS expression at day6 is 9.7±2.1 spots.aggr^{-1} or 293±64 spots.g^{-1} FM counted as transgenic unit (TU). Relative growth rate (RGR) is calculated as follow : FM_{day60}-FM_{day0}/FM_{day0}. No browned calli has been observed at day12.

Growth regulator concentration in medium of decontamination (mg.l^{-1})					Browning		RGR	GUS activity	
Timing of subculture (days)					(%)		(%)	(TU.aggr.$^{-1}$)	
D1-10	D10-20	D21-33	D34-49	D50-65	Day17	Day60	Day60	Day34	Day60
0.3	0.3	0.3	0.3	0.3	95	100	nd	nd	nd
1	0.3	0.3	0.3	0.3	86	100	nd	nd	nd
1	1	0.3	0.3	0.3	89	100	458	5.5±1.5^{ab}	3.3±1.2^{a}
1	1	1	0.3	0.3	89	100	1364	6.6±2.8^{a}	0.7±1.3^{b}
2	1	0.3	0.3	0.3	64	86	1585	3.3±1.5^{b}	0.6±0.5^{b}
2	2	1	0.3	0.3	39	60	2320	4±2.8^{ab}	0.3±0.4^{b}
2	2	2	1	0.3	39	23	3205	2.6±1.1^{b}	0.1±0.1^{b}

Fisher test (LSD). All values with the same letter are not significantly different (P<0.01).
Each value is the mean of 5 repetitions. Total of 20 tested aggregates.
Nd : not determined

Effect of growth regulators both concentration and timing in the decontamination medium on growth recovery of inoculated friable calli (W11)

The aim of this experiment is to get more details about the effect of GR in order to adjust accurately their concentration to keep positive effect on growth recovery of infected tissue and stability of the transgene expression.

Table 11 summarises the effect of growth regulators on the relative growth rate and the browning of infected tissue 33 days after co-cultivation. Growth recovery of cell aggregates after transformation is shown up by RGR, which increased with the GR concentration and their time of application before (pre-cultivation on 1 mg/l GR) and after co-cultivation. Nevertheless, RGR is optimal for an application of 2 mg/l GR for 20 days, beyond this period (33 days), the RGR tended to decrease. Besides, GR application reduced consequently the browning of culture.

Table 11. Effect of growth regulator (GR) concentration on the relative growth rate (RGR ; FM_t-FM_0/FM_0) and the browning of infected tissue.

Growth regulator concentration ($mg.l^{-1}$) Timing of subculture						Browning	RGR (%)		
Pre-culture	Medium of decontamination					(%)	Day6	Day18	Day33
	1-10	11-20	21-33	34-49	50-69	Day33			
0.3	0.3	0.3	0.3	0.3	0.3	94	Nd	7.85 ± 1.79^{b}	15.14 ± 3.76^{c}
1	0.3	0.3	0.3	0.3	0.3	97	2.74 ± 0.08^{b}	12.98 ± 5.01^{ab}	25.98 ± 11.05^{bc}
1	1	0.3	0.3	0.3	0.3	96	2.70 ± 0.42^{b}	14.34 ± 5.06^{a}	21.77 ± 5.06^{bc}
1	1	1	0.3	0.3	0.3	84	2.70 ± 0.42^{b}	10.33 ± 3.71^{ab}	24.02 ± 5.82^{bc}
1	1	1	1	0.3	0.3	67	2.70 ± 0.42^{b}	10.33 ± 3.71^{ab}	42.20 ± 11.35^{ab}
1	2	1	0.3	0.3	0.3	64	3.41 ± 0.38^{a}	12.91 ± 3.79^{ab}	39.17 ± 12.70^{ab}
1	2	2	1	0.3	0.3	60	3.41 ± 0.38^{a}	15.60 ± 5.78^{a}	50.35 ± 29.28^{a}
1	2	2	2	1	0.3	56	3.41 ± 0.38^{a}	15.60 ± 5.78^{a}	41.65 ± 14.96^{ab}

Fisher test (LSD). All values with the same letter are not significantly different ($P<0.01$).
Each value is the mean of 5 repetitions.
Nd : not determined (value higher than treatment with 1 mg GR, variation related to higher quantity of tissue transferred onto MD1)

GUS activity recorded as transgenic units (spot, cluster or cell mass) per initially cell aggregates transferred onto MD1 has been monitored for 2 months after the co-cultivation of friable calli with *Agrobacterium* cells (Table 12).
A low transformation has been initially recorded in comparison with other experiments. Significance of conclusions must be carefully taken into account. However, following trends might be described:

- At day6 browning of tissue already occurred but not yet degeneration. Thus GUS activity can be observed in all transgenic cells. This activity was decreased when 2 mg/l GR was applied and no difference of activity has been recorded between 0.3 and 1 mg/l,
- Later, GUS activity tended to decrease for all treatments except the ones in which cell aggregates were subcultured for 1 month onto medium containing 1 mg/l GR,
- The decrease was more important for treatments with 2 mg/l GR, and GUS activity reached zero for 1 month of application.

Despite the missing of significance of these data, we would like to put the following hypothesis:
Two phenomena might be emphasised to explain loss of GUS activity:

1- transgenic cells might degenerate when growth recovery after transformation is not sufficient,
2- transgene silencing might occur for 2 mg/l GR and increase with the duration of application.

These hypotheses are supported by the maintenance of the GUS activity in the treatments with 1 mg/l GR for 17 and 29 days (Table 12).

Table 12. Kinetics of GUS activity and relative growth rate of infected tissue cultivated onto medium containing 1 mg/l GR for 29 days. Relative growth rate (RGR; FM_t-FM_0/FM_0). Transgenic units (TU) consisted of both blue spots and cell clusters.

Initial FM	Relative growth rate (%)				GUS activity	GUS activity			
(mg)	Before selection			Selection	$TU.g^{-1}FM$	$TU.agg^{-1}$			
	Day6	Day18	Day33	Day67	Day6	Day6	Day18	Day33	Day67
11.1± 0.7	2.7±0.42	10.33±3.7	42.2±11.35	118.0±29.4	23.93±9.69	0.97±0.38	0.97±0.68	0.93±0.79	1.3±2.1

In conclusion, we propose to confirm the 2 hypothesis by surveying the effect of the concentration of growth regulators on the stability of GUS activity. The development of blue cell clusters and cell masses from blue spots gave evidence that transgenic cells have been able to divide. However, the loss of GUS activity occurring after this development must be explained to determine and find out this problem.
The transgene silencing hypothesis will be verified by application of an anti-methylation agent (5-azacytidine) in next experiment. Indeed, transgene silencing has been shown to be mediated by T-DNA methylation.

4.2.3 **Improvement of the decontamination of transformed tissues**

Effect of the antibiotics (N10)

Bacteriostatic antibiotics (cefotaxime and Ticarpen) were added to the decontamination medium in order to limit bacterial development during the culture phases, and their effects on tissue browning were also compared (Table 13). Ticarpen was found more effective than cefotaxime to control tissue browning observed 10 days after co-cultivation since about 30 % of the calli turned brown with Ticarpen instead of 100 % with cefotaxime. Interestingly, the addition of cefotaxime in the medium containing Ticarpen accelerated tissue browning when compared to Ticarpen alone. Nevertheless, Ticarpen alone could not stop tissue browning but only delayed it for about 10 days (27-32% at day10 and 81-93% at day19). This 9-day delay allowed the proliferation of new tissue on the initially transformed calli. The percentage of this secondary tissue increased with Ticarpen concentration to reach 47 % for 750 $mg.l^{-1}$ Ticarpen.
Cefotaxime has previously been shown to enhance regeneration in sweet sorghum (Rao et al., 1995) but to our knowledge, our report is the first emphasising the effect of cefotaxime on tissue browning. Cefotaxime might activate the metabolism and thereby induce tissue browning of culture in stress conditions such as those occurring upon transformation. Moreover, preliminary results showed that growth of non-transformed tissue could be stimulated by the addition of cefotaxime in the culture medium (data not shown). In any case, data reported here confirm observations that Ticarpen is an effective bacteriostatic agent and an alternative antibiotic for suppression of *Agrobacterium* (Cheng et al., 1998), and additionally demonstrate that it delays tissue browning when compared to cefotaxime. No contamination was observed in this particular trial but the control without antibiotics into the decontamination medium (data not shown), emphasising the variations sometimes observed regarding bacterial development.

Table 13. Effect of Cefotaxime and Ticarpen in the decontamination medium on browning of initially transformed tissue and calli growth recovery recorded as secondary calli growth. Tissue are subcultured as 5-6 mm cell aggregates after washing into 25 mg/l tetracycline solution for 20 min. and blotting for 30 min.

Antibiotics (mg/l)		Calli (N°)	Browning of primary tissue (%)		Presence of secondary tissue at day 34 (%)
Cefotaxime	Ticarpen		Day 10	Day 19	
0	0	54	72	100	0
250	0	66	100	100	4
500	0	66	100	90	8
750	0	35	100	95	6
0	250	60	32	93	24
0	500	61	30	84	27
0	750	64	27	81	47
250	250	66	85	88	8
500	250	61	97	72	8
250	500	66	89	74	23
500	500	54	91	78	15

4.3 Development of an adapted tissue culture system to transformed tissues

The transfer of big plant tissue aggregates implies a gradient of nutrition from the bottom of tissue to the top. Indeed, cells in contact with the medium have a better exchange with the medium components. When transformed tissues are transferred on decontamination medium, both bacteriostatic or selective agent (antibiotics) used respectively to inhibit *Agrobacterium* cell development and select plant transgenic cells, may not be able to act very well on all part of the tissue. Therefore, we assume that subculture of smaller pieces of tissue could favour a better both elimination of *Agrobacterium* cells and selection of transgenic cells.

Given the difficulties to cultivate small pieces of rubber tree tissues (preliminary experiments not shown), we initiated an approach gathering the simultaneous study of several factors such as container and tissue aggregates size, and timing of subculture. Given also that this study will serve to the culture of transformed tissues, we surveyed the influence of factors of pre-cultivation and decontamination.

Influence of the washing solution on tissue growth rate and browning (S1)

The effect of washing solution (water or MH: MM without GR) has been studied on non transformed tissues cultivated on maintenance medium (MM), washing being used for eliminating *Agrobacterium* cells from the plant tissue (Table 14, exp1). This varied according to the size of tissue aggregates. Washed tissues subcultured as small aggregates of 1-2 mm had the higher relative growth rate (RGR) above 100 in comparison with bigger sizes and the RGR reached a maximum at 160 for washing in water instead of 103 for washing in MH. On the contrary, when tissues were cultivated as bigger aggregates (3-4 or 5-6 mm), the use of MH (culture medium) is better. The washing in MH did not affect the RGR of the biggest aggregates (5-6 mm). However, RGR was reduced from 19 to 10 and 5 respectively for washing in water or the use of plate instead of bottle of 125 cc.
We have also to underline that the plating of tissues induced browning after one month of culture which was not observed for other culture conditions.

Influence of the culture container on tissue growth and browning (S2)

We first confirmed here that washing in water does not affect the RGR of tissues when they are subcultured as 1-2 mm cell aggregates and that these aggregates which have a higher RGR than those of 5-6 mm (Table 14, exp2). After washing in water, tissues were subcultured as 1-2 mm in various containers (plate, 125 cc or 250 cc bottle). RGR of aggregates cultivated in plate or 125 cc bottle were similar after 1 month of culture whereas the use of a bigger container, 250 cc, affected strongly the tissue growth after15 days of culture.

When tissues were washed in water supplemented with 250 mg/l cefotaxime and 250 mg/l Ticarpen, the RGR was reduced since the first 15 days of culture from 32 (control washed only with water) to 15 (this treatment). Therefore, the addition of bacteriostatic agents in the washing solution should be made with care and only if this appears absolutely necessary to eliminate *Agrobacterium* cells.

Besides, aggregates were subcultured on a nylon membrane, which is put on the culture medium in order to ease their transfer between each subculture and therefore reduce the handling stress. This culture system is usually favourable to the growth of tissues from several species such as rice, conifers, banana, etc. However, the use of nylon membrane affected dramatically the growth of rubber tree tissues, RGR decreased from 32 to 8, and tissue browning occurred within one month on 95% of cell aggregates. So this system has been abandoned.

Table 14. Effect of various culture conditions ($CaCl_2$ in pre-culture medium, washing (5 times 10 seconds) and blotting of transformed tissue (for 30 minutes on sterile Whatman paper), size of cell aggregates) on the tissue browning and the relative growth rate ($RGR = FM_{dayX} - FM_{day0} / FM_{day0}$)

Pre-culture	Tissue treatment			Cell aggregates		Browning (%)			RGR (%)	
$CaCl_2$ (mM)	Washing	Drying	Container	size (mm)	N°	Day8	Day15	Day32	Day15	Day32
Exp1										
9	-	-	125cc	5-6	38	0	0	0	Nd	24
9	-	-	Plate	5-6	38	0	0	0	Nd	19
9	H_2O	Blot	Plate	5-6	31	0	0	0	Nd	10
9	MH	Blot	Plate	5-6	43	0	0	3	Nd	17
9	H_2O	Blot	Plate	Plating	3 grams	0	0	29	Nd	5
9	H_2O	Blot	Plate	2-3	108	30	0	0	Nd	17
9	MH	Blot	Plate	2-3	115	0	0	0	Nd	32
9	H_2O	Blot	Plate	1-2	162	0	0	0	Nd	160
9	MH	Blot	Plate	1-2	143	0	0	0	Nd	103
Exp2										
9	-	-	125cc	5-6	29	0	0	0	10	40
9	-	-	Plate	1-2	125	0	0	0	17	72
9	-	-	125cc	1-2	72	0	0	0	17	91
9	H_2O	Blot	Plate	1-2	156	0	0	0	21	101
9	H_2O	Blot	125cc	1-2	142	0	0	0	32	93
9	H_2O	Blot	250cc	1-2	208	0	0	0	26	27
9	H_2O+ Cefo/Tim	Blot	125cc	1-2	139	1	2	Nd	15	Nd
9	H_2O	Blot	125cc	1-2/nylon*	142	1	8	95	8	Nd
Exp4										
9	-	-	Plate	1-2	50	0	0	0	Nd	99
0	-	-	Plate	1-2	50	0	?	0	Nd	85
9	-	-	Plate	3-4	50	0	0	0	Nd	39
0	-	-	Plate	3-4	50	0	0	1	Nd	39
9	H_2O	Blot	Plate	1-2	50	0	0	0	Nd	97
0	H_2O	Blot	Plate	1-2	50	0	0	0	Nd	94
9	H_2O	Blot	Plate	3-4	50	0	0	1	Nd	44
0	H_2O	Blot	Plate	3-4	50	0	0	2	Nd	50
9	MH_1^{4-}	Blot	Plate	1-2	50	0	0	0	Nd	106
0	MH_1^{4-}	Blot	Plate	1-2	50	0	0	0	Nd	107
9	MH_1^{4-}	Blot	Plate	3-4	50	0	0	2	Nd	45
0	MH_1^{4-}	Blot	Plate	3-4	50	0	0	1	Nd	40

* : 1-2 mm aggregates were transferred onto nylon membrane
Nd : data non determined

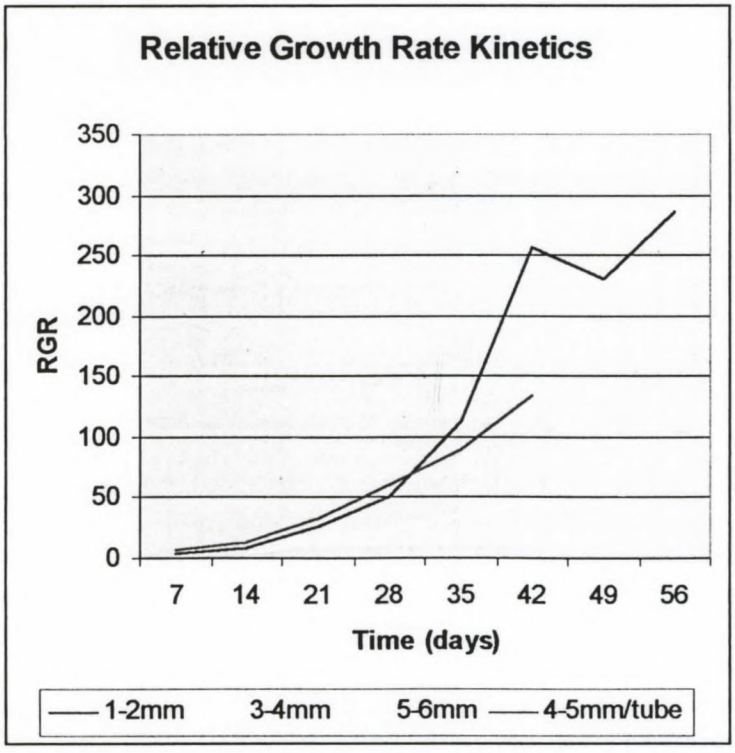
Influence of the $CaCl_2$ concentration in the pre-cultivation medium, the washing solution and the aggregate size on the tissue growth and browning (S3)

The use of a pre-culture phase of friable calli before transformation on a $CaCl_2$-less medium has been shown essential to get high transformation efficiency. This removal of $CaCl_2$ did not change the RGR and did not induce any tissue browning. We also confirmed in this experiment that on the one hand the washing conditions did not affect the same parameters, and on the other hand the 3-4 mm aggregates had a lower RGR than 1-2 mm whatever the conditions (Table 14, exp4).
Finally, we can conclude that in the case of non-transformation, the subculture of small aggregates of 1-2 mm is recommended to get a high growth rate of tissue. The washing of these tissues did not affect the tissue growth and could be used for eliminating Agrobacterium cells from transformed tissues.

Kinetics of relative growth rate in relation to the size of cell aggregates (S5)

The exponential growth phase is considered to be the time when cells are most active metabolically. This best timing is then chosen to transfer tissues onto fresh culture medium. Therefore, we drew the growth curve for each treatment. The relative growth rates were calculated in order to be able to compare growth rate between aggregates of various sizes.

Figure 1. Effect of the tissue aggregate size on the relative growth rate



The relative growth rate increased inversely with the size of aggregates when they are cultivated in bottle 125 cc (Figure 1). After 49 days of culture, a 1-2 mm aggregate initially weighing 1.8 mg can weigh 415mg and catch up bit by bit the weight of a 5-6 mm aggregates which weighed 63 mg at day0 and 567 mg at day 49.

The curves showed an exponential phase beginning at day20 for all conditions of culture. However, the curves for both 1 mm aggregates cultivated in bottle 125 cc and friable calli cultivated in tube sloped up rapidly, in contrast the 2 other conditions revealed a gentle slope to reach the stationary phase at day 30 and 45 respectively for 5-6 and 3-4 mm aggregates. For the 2 treatments with steep slope during exponential phase, friable calli cultivated in tube and those of small size in bottle 125cc, the stationary phase was reached after 40 to 50 days of culture.

4.4 Determination of culture conditions for selection (preliminary results).

Determination of the most suitable selective agent for selection of transgenic cells after transformation of Hevea friable calli (R6)

Two binary vectors bearing 2 different antibiotic resistance genes for selection of transformed plant cells, *nptII* (neomycin resistance) and *hgh* (hygromycin resistance), are available in our laboratory. This preliminary experiment aimed at determining the most efficient selective agent to block the non-transgenic cell proliferation from rubber tree tissue when friable calli are subcultured as small size cell aggregates.
Two neomycin antibiotics (kanamycin and paromomycin) and hygromycin have been attempted in this experiment.

Table 15. Effect of antibiotics on the relative growth rate (RGR), and tissue browning.

Antibiotics		Aggregates	Relative growth rate		Browning (%)		
Kind	Concentration				Primary tissue		All tissue
	(mg/l)	(No)	Day16	Day28	Day16	Day28	Day28
Control	0	75	4±1.2	18.5±4.4	0	32	0
Kanamycin	50	75	6.6±1.1	16.5±3	1	36	0
	100	75	11.6±2.4	18±3	0	22	0
	150	75	6.2±0.7	14.5±3.5	0	34	0
Paromomycin	50	75	3.1±1.1	11.2±1.8	7	100	0
	100	75	3.3±1.3	6.3±1.6	13	100	0
	150	75	0.7±0.5	1±0.2	77	100	100
Hygromycin	50	75	2.3±0.6	11.3±2.4	51	100	48
	100	75	1±0.2	2±0.6	48	96	100
	150	75	0.5±0.1	0.6±0.4	40	42	40

Kanamycin did not affect tissue growth at all. We must noticed that kanamycin stock has been found not effective (100 mg/l must be used to have a 50 mg/l similar effect). Paromomycin was more effective than kanamycin to decrease growth rate since 50 mg/l was used. Growth was totally blocked with 150 mg/l, and tissue browning increased with the antibiotic concentration after a15-day-culture. In the second subculture (Day15-day30), all primary transformed tissue turned brown.
Hygromycin acted at lower concentration and as soon as it was applied it blocked growth and induced browning. Nevertheless, data recorded at the browning level were totally illogical when we had less brown tissue with 150 mg/l than with 50 mg/l.
With regard to new growing tissue, hygromycin whatever its concentration and paromomycin with at least 150 mg/l could induce tissue browning. However, we have to notice a paradoxical result with 150 mg/l hygromycin that did decrease the percentage of brown tissue.

Kanamycin represents an ineffective antibiotic to select rubber tree transgenic cell, what it is amazing given 50 mg/l is commonly enough for most dicotyledon species.
Paromomycin is also a neomycin often use in liquid medium to select cell protoplast. This antibiotic is more effective to block rubber tree cell proliferation than kanamycin but necessitate 30-day-culture with high concentration 150 mg/l.
Hygromycin could be also used for selection but will require using EHA105pC1301 that contains *hgh* gene for hygromycin resistance.
Paromomycin and hygromycin will be attempted but we have to keep in mind than hygromycin induced more browning of non-transgenic cell that may affect transgenic cell proliferation.

Determination of the toxic paromomycin concentration for non-transformed tissue of Hevea friable calli (R9)

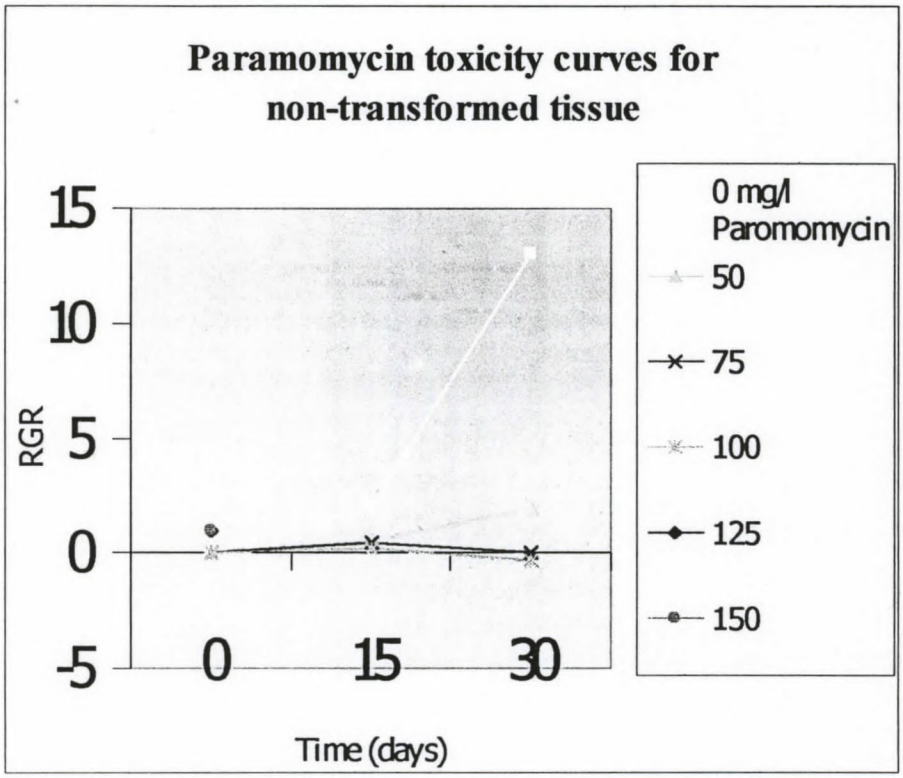
The preliminary experiment N6 aimed at determining the most efficient selective agent to block the non-transgenic cell proliferation from rubber tree tissue when friable calli are subcultured as small size cell aggregates. Kanamycin was shown totally ineffective but conversely both paromomycin and hygromycin have been confirmed very effective at high concentration.
Paromomycin has been attempted here because we routinely transformed with EHA105pC2301 that provides neomycin resistance.

Table 16. Effect of paromomycin on the relative growth rate (RGR), and tissue browning.

Paromomycin	Aggregates No	Relative growth rate		Browning %	
Concentration (mg/l)		Day16	Day28	Day16	Day28
0	100	2.03±0.50	12.97±5.28	0	0
50	100	0.51±0.42	1.92±1.78	6	11
75	100	0.70±0.32	0.62±0.31	30	29
100	100	0.43±0.17	-0.01±0.44	43	80
125	100	0.20±0.30	-0.36±0.26	53	92
150	100	0.01±0.08	-0.33±0.24	50	97

Growth rate and tissue browning were both affected by paramomycin (Table 16). RGR was dramatically decreased since 50 mg/l paromomycin was added (Figure2). For one month of antibiotic application, a loss of fresh matter and a strong tissue browning were observed beyond 100 mg/l. The tissue browning increased with the paromomycin concentration.

Figure 2. Paramomycin toxicity curves for non-transformed tissue from H. brasiliensis. The growth rate was measured in 5x5 pieces of tissue aggregate for each paromomycin concentration (0, 50, 75, 100, 125, 150).



Medium of decontamination supplemented with 50-75 mg/l paromomycin might be sufficient to block growth of non-transgenic tissue. Beyond 100 mg/l paramomycin, tissue was strongly affected with a high percentage of brown aggregates and a loss of fresh matter that might affect the development of transgenic cells as well.
Further experiments will be conducted on transformed tissue in order to take into account the transformation stress that must decrease the growth rate. Thus, the paromomycin concentration and its timing of application will be set up to select transgenic cells in an environment not to unfavourable.

Effect of both the washing and the aggregate size of the transformed tissue (R2)

Transformed tissues were transferred as cell aggregates of various sizes onto the decontamination medium supplemented with 4.8 µM GR for 15 days. They were then transferred on the same medium but with 1.4 µM GR. Beyond 26 days after co-cultivation, only transformed tissues transferred as 1-2 mm cell aggregates had grown (Table 17). Dramatic browning occurred regardless of the conditions when bigger aggregates were subcultured, and 60% of 5-6 mm aggregates were contaminated. Therefore GUS assays on 3-4 mm and 5-6 mm aggregates was not performed at day 26 and 59.
With respect to 1-2 mm aggregates, the number of blue units per aggregate could be monitored throughout the assay period and revealed a slight variation (significant at day 26) between washed and non-washed transformed tissues. A drop in GUS activity was recorded on transformed tissue washed after co-cultivation whereas transformed tissue directly subcultured without washing showed a slighter GUS activity decrease.

Moreover, non-washed tissues presented a higher relative growth rate (24±5 instead of 13±3) and lower tissue browning (8% instead of 34%).

Table 17. Effect of washing conditions and size of transferred cell aggregates on the GUS activity, relative growth rate (RGR), browning and contamination of transformed tissue. After a days of co-cultivation, transformed tissues were washed or not with a solution supplemented with 25 $mg.l^{-1}$ tetracycline for 20 min, and then blotted for 30 min. Tissues were transferred as 1-2, 3-4 or 5-6 mm cell aggregates onto MM supplemented with 500 $mg.l^{-1}$ cefotaxime.

Cell aggregates at day 0				GUS activity (blue units.agg^{-1})			Cell aggregates at day 26		
Washing	Size (mm)	FW ($mg.agg^{-1}$)	N°	Day6	Day26	Day 59	RGR	Browning (%)	Contamination (%)
+	1-2	1.8±0.3	168	1.1±0.3^{c}	0.3±0.4^{b}	0.1±0.1^{a}	13±3^{b}	34	0
-	1-2		194	Nd	0.8±0.4^{a}	0.5±0.6^{a}	24±5^{a}	8	0
+	3-4	39±13	60	5.7±1.7^{b}	Nd	Nd	Nd	100	0
-	3-4		60	Nd	Nd	Nd	Nd	100	0
+	5-6	333±47	30	38±22^{a}	Nd	Nd	Nd	100	60
-	5-6		30	63±30^{a}	Nd	Nd	Nd	100	62

Fisher test (LSD). All values with the same letter are not significantly different ($P<0.01$).
Each value is the mean of 5 repetitions. A total of 20, 30 and 150 aggregates respectively for sizes 5-6, 3-4 and 1-2 mm were tested for GUS assay.
Nd: not determined

The reduction in the size of transferred aggregates improved their growth recovery after transformation without the requirement for additional growth regulators. This confirms similar observations made on non-transformed tissues. Higher relative growth rates might be an advantage to overcome browning which occurs mostly in differentiated cells by the accumulation of oxidised polyphenols [2]. The optimisation of the tissue culture method, by allowing rubber tree transformed cell growth recovery after co-cultivation with *Agrobacterium*, removed the main constraint on the development of transgenic cell lines. In fact, growth recovery of transformed tissues before selection has been reported to be a critical step for the success of the selection of transgenic cells in larch [1], and in Norway spruce and loblolly pine [7]. The setting up of adapted conditions for selecting the transgenic rubber tree cells can be now considered.

5. INVOLVED SCIENTISTS AND HUMAN RESOURCE DEVELOPMENT

5.1 Research activities

Table 18. Presentation of the research activities per scientist

Name	Position	Assigned		Research programs at KAPI
		From	Until	
Pascal Montoro	CIRAD scientist	08.98	06.01	Setting up of the genetic transformation procedure
Nongluk Teinseree	DTEC assistant	08.98	07.99	
Reena Kanthapura	DTEC assistant	10.99	06.01	
Sukuntaros Tadakittisarn	KAPI scientist	06.99	12.99	Development of an adapted in-vitro system for selection of transgenic cell
Wiparat Rattana	KAPI scientist	10.98	09.00	Influence of exogenous growth regulators on cell competency to transformation and the growth recovery of transformed tissue
Waraporn Chorobchur	KAPI technician	10.98	09.00	Maintenance of plant tissue culture

5.2 Training

Table 19. Presentation of performed training

Name	Position	Training			
		Site	Period	Source of financement	Topic
Nongluk Teinseree	DTEC assistant	Cirad-France	25.02 to 05.04.99	Cirad grant (bourse hors champ)	Contributing of histological analyses of friable calli in genetic transformation of rubber tree
Wiparat Rattana	KAPI scientist	Cirad-France	01.09 to 15.10.00	French embassy grant	Influence of medium components on cytological characteristics of rubber tree tissues related to their competency to transformation and their ability to recover growth after transformation
Poom Pimprapan	Master student	Kapi	10.98 to 09.00	-	Genetic transformation of tobacco and rubber tree friable calli
Tanapon Glingaysorn	Master student	Kapi	06.99 to 10.99	-	Genetic transformation of tobacco and rubber tree friable calli

Up to 5 persons have participated to this research program conducted at Kapi. The scientists were trained in the Kapi laboratory on:

- tissue culture of rubber tree and tobacco;
- microbiology;
- *Agrobacterium tumefaciens*-mediated genetic transformation;
- histochemical GUS assay;
- cytological observation.

In terms of human resource development, 2 students and 2 scientists performed a training respectively in the Kapi and Biotrop Laboratory at Cirad in Montpellier.

5.3 Publication in preparation

The following manuscript will be submitted at Plant Cell Tissue and Organ Culture for publication. This paper summaries a part of the studies carried out in this present project:

"Characterisation of factors involved in tissue growth recovery and stability of GUS activity in rubber tree (*Hevea brasiliensis*) friable calli transformed by *Agrobacterium tumefaciens*"

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Abstract

The influence of various parameters on *Agrobacterium tumefaciens*-mediated gene transfer in *Hevea brasiliensis* friable calli was investigated. Transient GUS activity is more dependent on the duration of the co-cultivation step than the inoculum concentration. Washing of the transformed tissues in a solution supplemented with tetracycline reduced GUS activity probably reflecting transformation events occurring after co-cultivation. Replacement of cefotaxime by timentin in the decontamination

medium delayed tissue browning and allowed part of the transformed tissue to proliferate. Growth recovery of the whole transformed tissue was stimulated when higher concentrations of growth regulators were introduced in the culture medium following co-cultivation. Adversely, the stability of the GUS activity was affected by these modifications in the growth regulators concentration and timing of application. The transfer of smaller pieces of non-washed transformed tissues onto the decontamination medium further improved the growth and allowed maintenance of the GUS activity.

6. CONCLUSION

Taking advantage of the high transformation efficiency conditions set up in rubber tree friable calli, the experiments presented in this report dealt with the elimination of the *Agrobacterium* cells and the mean to boost growth recovery of plant cell after transformation while maintaining a sufficient gene transfer efficiency.
In that way, we tried to decrease biotic stress. It appeared that first, the use of the strain EHA105, and second, the balance between the *Agrobacterium* cell concentration in the suspension for inoculation and the time of co-cultivation, were important factors to maintain sufficient transformation efficiency to consider further development of transformed tissues. Nevertheless, browning occurred systematically after a while in transformed tissue cultivated in previous conditions, 5-6 mm aggregates and decontamination supplemented with 0.3 mg/l growth regulators (GR).
Only a stimulation by higher growth regulator concentration allowed a development of transformed tissues without browning. However, the GUS activity in these tissues in proliferation decreased over the culture with the GR concentration. Although, transgenic cell division has been observed at the beginning of the culture, the proliferation of transgenic tissues stops and might explain the loss in GUS activity.
In parallel, a new tissue culture system has been developed. Small tissue aggregates cultivated in bottle of 125 cc have a very high relative growth rate. In addition, the small aggregates have a better contact with the medium and subsequently bacteriostatics present in the decontamination medium were shown to inhibit strongly the *Agrobacterium* cell development ; thereby the washing of transformed tissues after co-cultivation which is considered as water stress step has been removed.
Preliminary experiments on selection step showed that kanamycin is ineffective to select *Hevea* transgenic cells. In contrast, paromomycin inhibits the proliferation of non-transgenic after 1 month. Use at 50 mg/l, the paromomycin did not provoke any strong tissue browning. This is assumed as an essential condition because we know that in rubber tree tissue, browning propagation occurred rapidly. Therefore, that could help to the transgenic cell proliferation by maintaining a better environment.

7. SHORT TERM PROSPECTS

GUS activity has been monitored in transformed tissues. After several month of selection, transgenic cells were observed as blue cell clusters but rarely as tissue in proliferation. The low proliferation rate of transgenic cells appears now as the main constraint to establish transgenic cell lines which will be used to regenerate transgenic plants. Cytological studies are under way in order to characterise the influence of medium components on the cell activity and then to determine conditions allowing the development of transgenic cells.

A series of experiment will be initiated in order to maintain the cell activity in a good environment favourable to the transgenic cell proliferation. If transgenic cell lines are established, we will finally perform a molecular characterisation of these lines. Besides, we will control the maintenance of the embryonic capability of transformed tissues.

8. REFERENCES

1. Levée V, Lelu M-A, Jouanin L, Cornu D, Pilate G: *Agrobacterium tumefaciens*-mediated transformation of hybrid larch (*Larix kaempferi x L. decidua*) and transgenic plant regeneration. Plant Cell Reports 16: 680-685 (1997).
2. Michaux-Ferriere N, Carron MP: Histology of early somatic embryogenesis in *Hevea brasiliensis* : the importance of the timing of subculturing. Plant Cell, Tissue and Organ Culture 19: 243-256 (1989).
3. Montoro P, Pujade-Renaud V, Teinseree N: Strategy to study functionality of putative promotor from *Hevea brasiliensis* : attempts of *Agrobacterium tumefaciens* -mediated gene transfer in various tissues. The Biochemical and Molecular Tools for Exploitation and Diagnostic and Rubber Tree Improvement., Bangkok, Thailand (1997).
4. Montoro P, Teinseree N, Rattana W, Kongsawadworakul P, Michaux-Ferriere N: Effect of exogenous calcium on *Agrobacterium tumefaciens* -mediated gene transfer in *Hevea brasiliensis* (rubber tree) friable calli. Plant Cell Reports 19: 851-855 (2000).
5. Montoro P, Teinseree N, Rattana W, Tadakittisarn S: Genetic transformation of rubber tree. Annual report 1998-99. DORAS, Bangkok (1999).
6. Nongluk: Contributing of histological analyses of friable calli in genetic transformation of rubber tree (Hevea brasiliensis Müll. Arg), pp. 20. CIRAD, Montpellier (1999).
7. Wenck AR, Quinn M, Whetten RW, Pullman G, Sederoff R: High-efficiency Agrobacterium-mediated transformation of Norway spruce (Picea abies) and loblolly pine (Pinus taeda). Plant-Molecular-Biology. 39: 407-416 (1999).